Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells

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Centrosome duplication is indispensable for the formation of the bipolar mitotic spindle. Surprisingly, even if DNA replication or mitosis is inhibited, centrosome duplication can still occur [1–5]. Thus, it remains unknown how centrosome duplication is coordinated with the cell cycle. Here, we show that centrosome duplication requires cyclin-dependent kinase 2 (Cdk2) in mammalian cells. We have found that in Chinese hamster ovary (CHO) cells, whereas centrosome duplication is not inhibited by hydroxyurea (HU) treatment, which arrests the cells in S phase, it is inhibited by mimosine treatment, which arrests the cells in late G1 phase. Cdk2 activity was higher in HU-treated cells than in mimosine-treated cells. Remarkably, inhibition of the Cdk2 activity in HU-treated cells with butyrolactone I or roscovitine [6], or by expression of the Cdk inhibitor $p21^{Waf1/Cip1}$, blocked the continued centrosome duplication. Moreover, overexpression of Cdk2 reversed the inhibition of centrosome duplication by mimosine treatment. These results indicate a requirement of Cdk2 activity for centrosome duplication and therefore suggest an underlying mechanism for the coordination of centrosome duplication with the cell cycle.

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Results and discussion

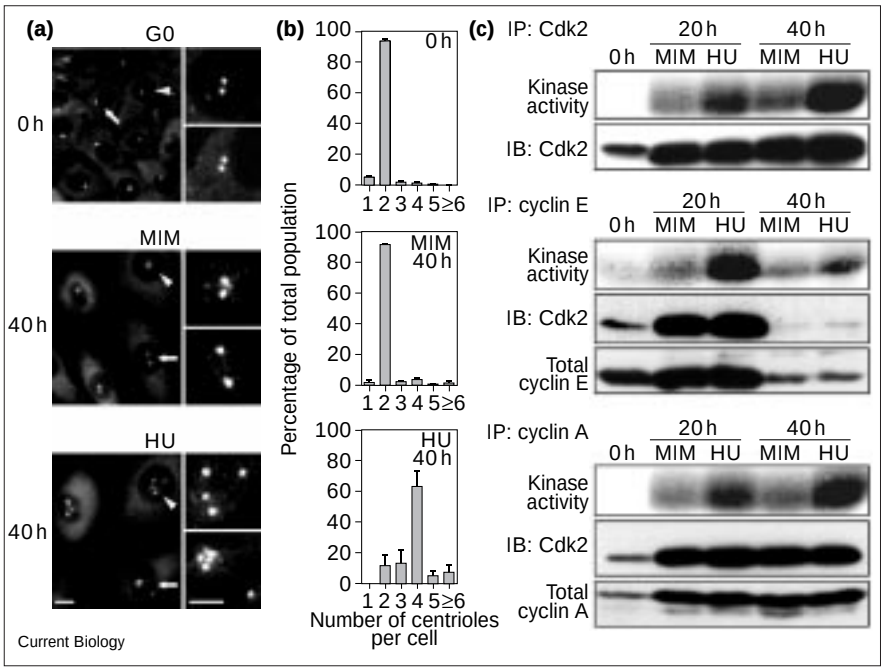
Centrosome duplication is known to repeat during the prolonged early S phase induced by treatment of CHO cells with HU [4]. We first compared the effect of two cell cycle inhibitors, mimosine and HU, on centrosome duplication in CHO cells. Mimosine has been shown to inhibit the cell cycle in late G1 phase [7]. Cells were arrested in quiescent state (G0) and then induced to enter the cell cycle by the addition of serum. Quiescent cells contained one centrosome consisting of two centrioles (Figure 1a, 0 h). In the presence of 2 mM HU, centrosome duplication is not blocked. About 50–60% of cells treated with HU for 40 hours had more than two centrioles per cell (which were clearly discernible), but about 30% of the cells had packed centrioles, the number of which could not be counted accurately. We therefore treated the cells with 0.5 μM okadaic acid (OA) for 40 minutes to separate these packed centrioles for accurate quantitation. After the OA treatment, about 88% of the cells contained more than two centrioles per cell (Figure 1b, HU 40 h). Using electron microscopy, we verified that centrosome duplication occurred without the OA treatment (see Supplementary material published with this paper on the internet).

The effect of mimosine on centrosome duplication was different from that of HU. In the presence of 0.4 mM mimosine, which inhibited S phase entry (data not shown), only two centrioles were detected in more than 90% of the cells even 40 hours after serum addition (Figure 1a,b). Thus, no sign of centrosome duplication was observed in the presence of mimosine. We confirmed this by using electron microscopy (see Supplementary material). These results may suggest that centrosome duplication is dependent on some event occurring at the G1–S boundary.

Cdk2 is known to be activated as a Cdk2–cyclin E complex at the G1–S boundary and as a Cdk2–cyclin A complex during S phase progression [8]. We therefore examined Cdk2 activity in CHO cells treated with mimosine or HU. Lysates from cells treated with mimosine or HU were subjected to an immunocomplex kinase assay for Cdk2 with histone H1 as a substrate. In the presence of HU, Cdk2 activity increased significantly after the serum addition. In the presence of mimosine, however, the increase in Cdk2 activity was significantly inhibited (Figure 1c). Both the cyclin-E-associated H1 kinase activity and the cyclin-A-associated H1 kinase activity were much lower in the presence of mimosine than in the presence of HU (Figure 1c). Thus, mimosine, but not HU, arrests the cell cycle before the rise in the activity of Cdk2, as suggested previously [9]. We therefore hypothesized that centrosome duplication must depend on the activity of Cdk2.

To test this hypothesis, we examined whether inhibition of Cdk2 activity suppresses the repeated centrosome duplication in the presence of HU. To inhibit Cdk2 activity, we used two drugs, butyrolactone I and roscovitine, both of which are reported to inhibit specifically the kinase activity of Cdk2, Cdc2 or Cdk5 [6]. In a control experiment in which dimethylsulfoxide (DMSO) was added (the solvent for the drugs), about 77% of the cells contained

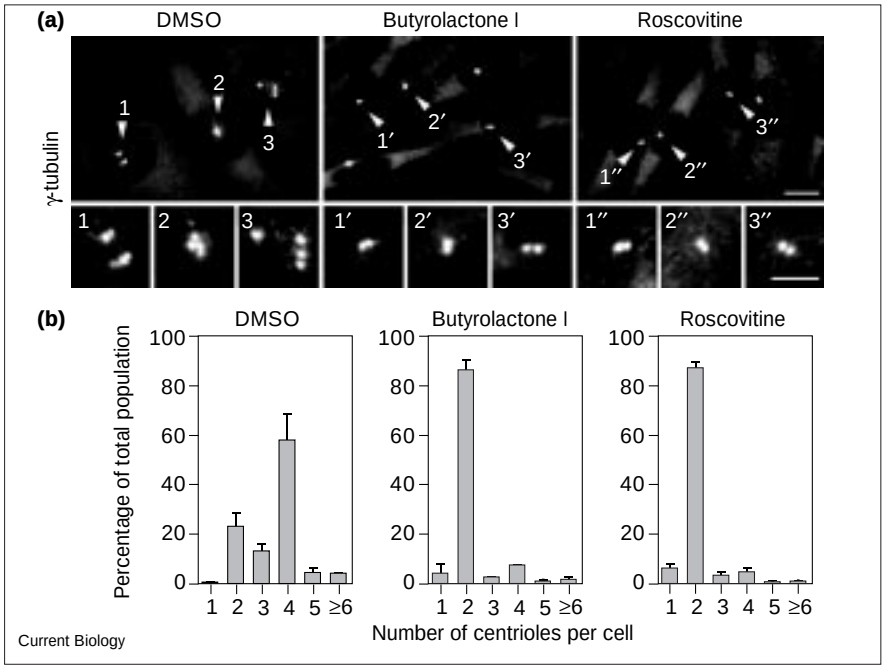
Figure 1



Centrosome duplication is inhibited by mimosine treatment but not by HU treatment. (a) CHO cells were serum starved (0.2% fetal calf serum) for 48 h, split onto collagen-coated glass coverslips at about 50% confluency, then treated with 400 μM mimosine (MIM) or 2 mM HU, together with 10% fetal calf serum, and incubated for 40 h. The cells were treated with OA and stained with anti-γ-tubulin antibody. Cells arrested in the quiescent state (G0) are also shown (0 h). The scale bar represents 10 μm. Magnified images of the centrioles indicated by the arrowheads and arrows are shown in the upper and lower insets, respectively; here, the bar represents 5 μm. (b) Quantification of the number of centrioles per cell in the cells treated with mimosine or HU for 0 h and 40 h. More than 200 cells were counted in one experiment and data are shown as the mean ± standard deviation (error bars) of three independent experiments. (c) Cdk2 activity in the cells treated with mimosine or HU. Lysates were obtained from mimosine-treated or HU-treated cells (at 0 h, 20 h and 40 h) and subjected to immunoprecipitation with anti-Cdk2 (IP: Cdk2), anti-cyclin E (IP: cyclin E) or anti-cyclin A (IP: cyclin A) antibodies. The immunoprecipitates were assayed for kinase activity, and immunoprecipitates from 200 μg lysate protein were immunoblotted with anti-Cdk2 antibody (IB: Cdk2). Total lysates (50 μg protein) were immunoblotted with anti-cyclin E (total cyclin E) or anti-cyclin A (total cyclin A) antibodies.

more than two centrioles per cell in the presence of HU. In contrast, 85.7% of butyrolactone-I-incubated cells and 86.6% of roscovitine-incubated cells still had only two centrioles per cell (Figure 2). Thus, both butyrolactone I and

Figure 2



Butyrolactone I or roscovitine inhibits the continued centrosome duplication in HU-treated cells. (a) Cells were treated with HU for 10 h and then an equal amount (7.2 μl per 2 ml medium) of DMSO, butyrolactone I stock solution or roscovitine stock solution (the final concentration of butyrolactone I or roscovitine was 180 μM) was added. Cells were incubated for a further 10–40 h and then the drugs were removed before OA treatment and staining with anti-γ-tubulin antibody. The bar represents 10 μm. The centrioles indicated by the numbered arrowheads are shown as magnified images in the correspondingly numbered insets; here, the bar represents 5 μm. (b) Quantification of the number of centrioles per cell in the cells incubated with DMSO, butyrolactone I or roscovitine in the presence of HU. More than 200 cells were counted in one experiment and data are shown as the mean ± standard deviation of three independent experiments.

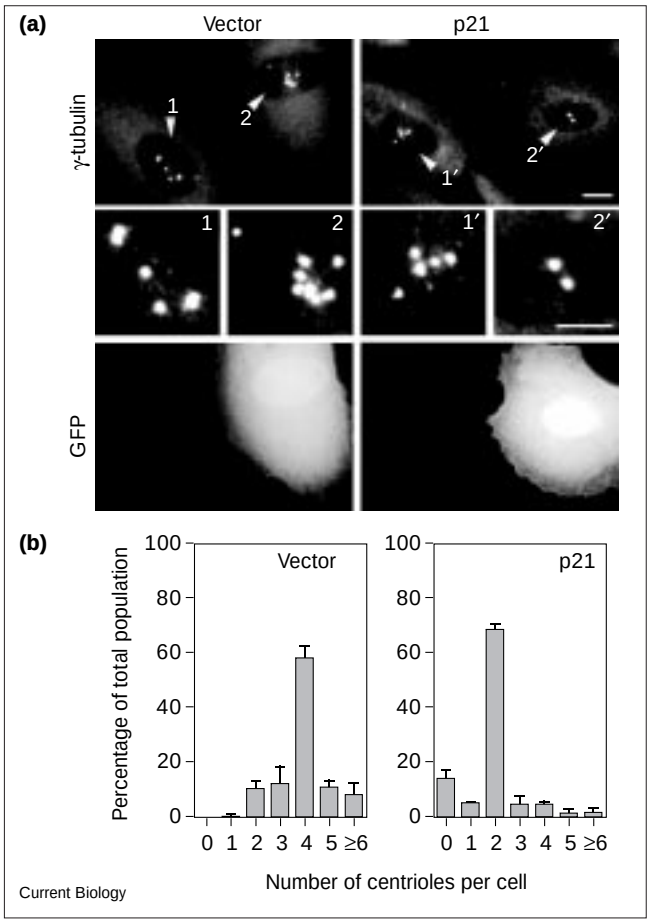
roscovitine strongly inhibited the continued centrosome duplication in the presence of HU.

Next, we expressed the Cdk inhibitor $p21^{Waf1/Cip1}$ (p21) in HU-treated CHO cells to inhibit the activity of Cdk2. We transfected a p21 expression plasmid or empty vector together with a green fluorescent protein (GFP) expression plasmid as a transfection marker. By immunostaining with anti-p21 antibody, we confirmed that p21 and GFP were co-expressed (data not shown). Most of the cells transfected with empty vector (about 89%), like non-transfected cells, contained more than two centrioles per cell. In contrast, the majority of the p21-expressing cells (about 68%) had only two centrioles per cell (Figure 3). Taken together, these results indicate that Cdk2 activity is required for centrosome duplication.

To test whether an increase in Cdk2 activity reverses the inhibitory effect of mimosine treatment on centrosome duplication, we overexpressed Cdk2 in mimosine-treated cells. We transfected wild-type Cdk2 or kinase-dead Cdk2 (a single amino acid substitution of Thr160 for alanine) [10] expression plasmids, or empty vector, together with GFP plasmid. By immunostaining with anti-Cdk2 antibody, we confirmed that Cdk2 and GFP were co-expressed (data not shown). The majority of the cells transfected with either the empty vector or the kinase-dead Cdk2 (about 77%) had only two centrioles per cell in the presence of mimosine. In contrast, most of the cells expressing wild-type Cdk2 (about 71.7%) contained more than two centrioles per cell in the presence of mimosine (Figure 4a,b). Using electron microscopy, we confirmed that centrosome duplication actually occurred in the cells expressing wild-type Cdk2. We identified GFP-expressing cells on CELLocates (Eppendorf) before fixation. In four cells examined that expressed wild-type Cdk2, three cells contained four centrioles per cell and one contained three centrioles; these cells had normal triplet microtubules (Figure 4c). Only two centrioles per cell were seen in all the cells examined that had been transfected with either empty vector or the kinase-dead Cdk2 plasmid (Figure 4c). The H1 kinase activities of the immunoprecipitates obtained with anti-Cdk2, anti-cyclin E or anti-cyclin A antibodies were significantly higher in the cells expressing wild-type Cdk2 than in the cells transfected with empty vector or the kinase-dead Cdk2 plasmid (see Supplementary material). Thus, the increased H1 kinase activity of Cdk2 seems to be associated with both cyclin E and cyclin A. All these results clearly indicate that an increase in Cdk2 activity is able to reverse the inhibition of centrosome duplication induced by mimosine treatment and therefore support our idea that Cdk2 activity is required for centrosome duplication.

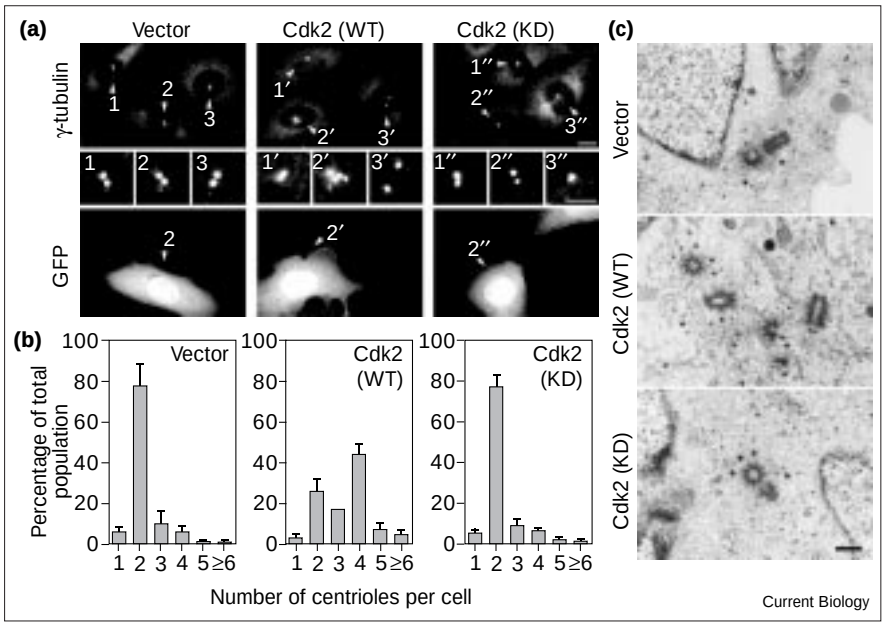
Our results strongly suggest that centrosome duplication is under the control of Cdk2. This enzyme has been previously shown to control DNA replication [8]. Therefore, we propose that activation of Cdk2 is the mechanism that ensures the concurrence of centrosome duplication and DNA replication. The continued centrosome duplication in the absence of DNA replication [1,4,5] is likely to be due to the relatively high Cdk2 activity in the prolonged S phase that occurs under these conditions. In the absence of protein synthesis, cells cannot enter mitosis because of the lack of mitotic cyclins, but centrosome duplication continues in the early embryos of the frog [3] and sea urchin [2] though does not continue in somatic cells [11]. Oscillations in the activity of Cdk2 are independent of protein

Figure 3



Expression of p21 inhibits the continued centrosome duplication in HU-treated cells. (a) Empty vector or a p21 expression plasmid, together with a GFP plasmid, were transfected into serum-starved cells 24 h before the addition of serum. The cells were then treated with 2 mM HU for 40 h and stained with anti-γ-tubulin antibody (γ-tubulin). Transfected cells were detected as those expressing GFP (GFP). The bar represents 10 μm. The centrioles indicated by the numbered arrowheads are shown as magnified images in the correspondingly numbered insets; here, the bar represents 5 μm. (b) Quantification of the number of centrioles per cell in the cells transfected with empty vector and in those expressing p21. More than 200 cells were counted in one experiment and data are shown as the mean ± standard deviation of three independent experiments.

Figure 4



Expression of Cdk2 reverses the inhibition of centrosome duplication in mimosine-treated cells. (a) Empty vector, or wild-type (WT) or kinase-dead (KD) Cdk2 expression plasmids, together with GFP plasmid, were transfected into serum-starved cells 24 h before the addition of serum. The cells were then treated with 0.4 mM mimosine for 40 h and stained with anti-γ-tubulin antibody (γ-tubulin). Transfected cells were identified as those expressing GFP (GFP). The bar represents 10 μm. The centrioles indicated by the numbered arrowheads are shown as magnified images in the correspondingly numbered inset; here, the bar represents 5 μm. (b) Quantification of the number of centrioles per cell in the cells transfected with empty vector and in those expressing wild-type Cdk2 or kinase-dead Cdk2. More than 200 cells were counted in one experiment and data are shown as the mean ± standard deviation of three independent experiments. (c) Transmission electron microscopy analysis of the cells transfected with empty vector and those expressing wild-type Cdk2 or kinase-dead Cdk2. The bar represents 400 nm.

synthesis during the early embryonic cell cycles in *Xenopus* [12]. In contrast, the formation of active Cdk2–cyclin E complex in somatic cells requires de novo synthesis of cyclin E [13]. Moreover, abnormal centrosome duplication is a possible cause of the genomic instability frequently seen in cancer cells [14,15]. Centrosome duplication is possibly restricted to one cycle by the proper control of Cdk2 activity. In conclusion, we have demonstrated the dependence of centrosome duplication on the Cdk2 cell cycle engine in mammalian cells. Therefore, our findings suggest an underlying mechanism for the coordination of centrosome duplication with the cell cycle. After submission of this work, a paper appeared that reports a similar observation in *Xenopus* egg extracts [16].

Supplementary material

Additional methodological details and two figures showing transmission electron microscopy analysis of cells treated with mimosine or HU and immunocomplex kinase assays of Cdk2-overexpressing cells are published with this paper on the internet.

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